Institut für Labortierkunde
der Vetsuisse-Fakultät Universität Zürich

Direktor Institut für Labortierkunde: Prof. Dr. Thorsten Buch

und

Zentrum für Klinische Forschung und Zentrum Chirurgie
Abteilung Forschung Chirurgie des Universitätsspitals Zürich

Leitung Abt. Forschung Chirurgie USZ: Prof. Dr. med. Rolf Graf

Arbeit unter wissenschaftlicher Betreuung von PD Dr. med. vet. Alessandra Bergadano,
dipl. ECVAA, PhD, Comparative Medicine, Roche Innovation Center Basel

Pharmacokinetics of tramadol hydrochloride after administration via different routes in male and female B6 mice

Inaugural-Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

vorgelegt von

Rocio Evangelista Vaz

Tierärztin

aus Spanien

genehmigt auf Antrag von

Prof. Dr. med. vet. Margarete Arras, dipl. ECLAM, Hauptreferentin
Prof. Dr. med. vet. Claudia Spadavecchia, dipl. ECVAA, PhD, Korrefentin

Zürich, 2016

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R. Evangelista Vaz, D.I. Draganov, C. Rapp, F. Avenel, G. Steiner, M. Arras, A. Bergadano

Title: Pharmacokinetics of tramadol hydrochloride after administration via different routes in male and female B6 mice.

1.Abstract

Objective: 1) Determine the pharmacokinetics of tramadol hydrochloride and its active metabolite, O-desmethyl-tramadol (M1) after administration through different routes in female and male C57Bl/6 mice. 2) Evaluate the stability of tramadol solutions.

Methods: Mice received 25 mg kg-1 tramadol as bolus [intravenously (IV), intraperitoneally (IP), subcutaneously (SQ), orally per gavage (OSgavage)] or over 25 h [oral in drinking water (OSwater) or Syrspend®SF (OSSyrsp)]. Venous blood was sampled at predetermined time points to determine tramadol and M1 plasma concentrations (LC-MS/MS detection). Pharmacokinetic parameters were described using a non-compartmental model. The stability of tramadol in water (acidified and untreated) and Syrspend®SF (0.20 mg mL-1) at ambient conditions for 1 week was evaluated.

Results: Tramadol showed low oral bioavailability (26%). After all administration routes Cmax of both tramadol and M1 were high (>100 ng mL-1 and >40 ng mL-1, respectively) and followed by short half-lives (2-6h). Plasma concentrations of tramadol and M1 after self-administration remained stable throughout consumption time; except for M1 in Sysrpend®SF group. Short-lasting side effects were observed after IV. Water and Syrspend®SF solutions are stable for 1 week.

Tramadol SQ (25 mg kg-1) followed by tramadol in water (25 mg kg-1 in 24 h) achieves plasma levels warranting efficacy assessment in further studies.

Keywords pharmacokinetics, analgesia, mice, tramadol.

Zusammenfassung

Ziele: Bestimmung der Pharmakokinetik von Tramadol-Hydrochlorid und aktiven Metaboliten M1 nach Verabreichung über verschiedene Applikationsarten an männliche und weibliche C57BL/6-Mäuse. Bewertung der Stabilität von Tramadol-Lösungen Wasser und Syrspend®SF bei Raumtemperatur über eine Woche.

Methoden: Den Mäusen wurde 25 mg kg^{-1} Tramadol als Bolus (Intravenös , Intraperitoneal, Subkutan, oral per Gavage) oder über 25 Stunden hinweg Oral in Trinkwasser oder in Syrspend®SF verabreicht. Die Plasmakonzentrationen von Tramadol und M1 wurden bestimmt (Flüssigchromatographie-Massenspektrometrie). Die Pharmakokinetik-Parameter wurden mithilfe eines nicht-kompartimentellen Modells beschrieben.

Ergebnisse: Tramadol hat eine geringe Bioverfügbarkeit (26%). Bei den gewählten Applikationsarten war Cmax für T und M1 hoch (>100 ng mL-1, bzw. >40 ng mL-1) und zeigten kurze Halbwertszeiten (2-6 Stunden). Die Plasmaspiegel von T und M1 nach Selbstapplikation blieben während der gesamten Aufnahmezeit stabil, mit Ausnahme von M1 in der Sysrpend®SF-Gruppe. Kurz-anhaltende Nebenwirkungen wurden nach IV-Applikation beobachtet. Wasser- und Syrspend®SF-Lösungen sind für eine Woche stabil.

Durch subkutane Applikation von Tramadol gefolgt von Tramadol in Wasser (25 mg kg^{-1} in 24 Stunden) können Plasmawerte erreicht werden, welche in einer Wirksamkeitsbewertung in späteren Studien überprüft werden sollten.

Schlagwörter: Pharmakokinetik, Analgesie, Mäuse, Tramadol

2. Manuscript

2.1 Introduction

Mice are widely used as laboratory models for surgical procedures. The provision of appropriate analgesia for peri- and postoperative pain is an ethical and legal imperative (Carbone 2011) and essential for scientific integrity as untreated pain is expected to affect the outcome data. However, providing an effective analgesic treatment for the target species is challenging for involved scientists (veterinarians, researchers, animal welfare bodies…) due to the biological peculiarities, the sparse published data of both the pharmacokinetics and efficacy of potentially relevant analgesics in the target species or strain, and finally the potential for interaction with the experimental read out.

Mice as a prey species tend to hide signs of pain, which hampers the recognition and quantification of pain, contributing to the underuse of postoperative analgesics. To date, the spectrum of analgesics available for laboratory mice relies mainly on few opioids (i.e. buprenorphine) and NSAIDs (carprofen, meloxicam). While offering potentially good analgesic options for mice (Tubbs et al. 2011; Oyama et al. 2012; Jirkof et al. 2015), they have limitations. NSAIDs are accompanied by anti-inflammatory and immune-modulatory effects (Iñiguez et al. 1999; Paccani et al. 2002), hence, inappropriate for studies involving inflammation and the immune system. Additionally their efficacy is questionable based on latest evidence (Roughan et al. 2016).

The μ-agonist opioids, apart from interfering with the immune response to some extent (Page 2005; Franchi et al. 2007; Ricardo Buenaventura et al. 2008), present dose-dependent undesirable side effects, such as respiratory and gastrointestinal depression, tolerance/hyperalgesia or increased activity (Flecknell 1984; Hayes et al. 2000; Hau & Schapiro 2002; Ricardo Buenaventura et al. 2008; Grimm et al. 2015).
Tramadol is a centrally acting synthetic opioid, structurally related to morphine and codeine (Kayser et al. 1992; Cannon et al. 2010; Zhang et al. 2014) used for the treatment of moderate to severe pain, both acute and chronic, in various species, including man (Lewis & Han 1997). Its analgesic efficacy is dependent on a complex set of interactions between opioid, adrenergic and serotonin receptor mechanisms: as an opioid agonist, tramadol has some selectivity for the μ-receptor and binds weakly to κ- and δ-receptors; furthermore, it also activates the monoaminergic system, inhibiting the neuronal reuptake of serotonin (5-hydroxytryptamine; 5-HT) and noradrenaline (NA; norepinephrine) (Driessen & Reimann 1992; Sacerdote et al. 1997). Both opioid and monoaminergic systems are deeply involved in the modulation and inhibition of pain. The clinical efficacy of tramadol is highly related to its metabolism but among its over 20 well-known metabolites, only one has analgesic properties, the *O-desmethyl-tramadol hydrochloride* (M1), which has 200-fold higher affinity for μ-receptors and up to 6-fold higher analgesic potency than tramadol itself (Raffa et al. 1993; Vullo et al. 2014). As a result of its composite mechanism of action, tramadol at clinical doses does not induce respiratory depression or hemodynamic changes, common to other opioids with a higher μ receptor activity (Lewis & Han 1997; Grond et al. 1999; Rätsep et al. 2013). In mice, central nervous system side effects have been reported such as restlessness or straub-tail (>10mg kg^{-1} IV) (Von G. Osterloch 1978; Matthiesen et al. 1998) and seizures at high doses (>80mg kg^{-1} IP) (Raffa & Stone 2008).

Due to its relatively high benefit/risk ratio, favorable pharmacokinetic properties, low potential for drug interactions in humans and other animal species (Lewis & Han 1997), and non-controlled substance schedule, tramadol might be an interesting candidate to widen the analgesic portfolio in mice. Actual evidence of the analgesic efficacy of tramadol in mice is

controversial: a recent study demonstrated that tramadol ameliorates cyclophosphamide-induced bladder-pain-related behaviors in mice (3-10 mg kg^{-1} orally given) (Oyama et al. 2012) , while (Wolfe et al. 2015) do not recommend it as a sole analgesic after abdominal laparotomy in mice. However these studies had no PK profiles supporting the dynamic data.

Usually in this species, analgesics are administered via subcutaneous (SQ) and intraperitoneal (IP) routes, a practice that may induce handling stress when carried out several times a day to ensure a seamless analgesia over time (Sharp et al. 2002). Pain treatment can be further improved by optimizing the methods of administration, (i.e. sustained/controlled release formulations or self-administration methods). Self-administration methods are an attractive option because they could ensure stable drug levels in the blood, which is necessary for adequate (in both intensity as duration) analgesic coverage while avoiding repetitive handling of the animals. Self-administration would resolve the stress caused by the repeated injection of drugs at defined intervals, depending on the species-specific pharmacokinetics. Therefore, in recent years self-administration methods using different types of vehicles (water, pellets, Nutella®, jelly) have been tested for oral drug delivery in laboratory animals with some success. There are already available data supporting this route of administration for analgesics (i.e. buprenorphine) in rats and mice (Abelson et al. 2012; Molina-Cimadevila et al. 2014); however, to the best of our knowledge, there is no published data regarding tramadol delivery in mice by such means.

Based on the rationales expressed and knowledge gaps in the literature, this study aims to: 1) Determine the pharmacokinetics of tramadol and M1 after tramadol administration through different routes in female and male B6 mice. 2) Evaluate the stability of tramadol in aqueous solution, to explore the feasibility of using drinking water for tramadol delivery. 3) Determine the most suitable route or combination of routes for this strain.

2.2 Materials and Methods

Animals

The experimental protocol was approved by the local veterinary authorities. Eighteen male and 18 female C57BL/6J mice (20-30 g body weight, BW) were used in this study. Inclusion criteria were: strain, age and BW, healthy on clinical examination and based on review of health reports (according to FELASA health monitoring recommendations) (Mähler et al. 2014). Exclusion criteria were: failure to adhere to pre-test requirements or overt sign of illness. Mice were housed in groups of three animals in standard polycarbonate cages, with aspen wood bedding (J. Rettenmeier & Söhne GmbH, Germany) and nesting material; a rotational enrichment plan was in place, with hemp rope (Cordag AG, Switzerland) and aspen wood stick (LAB & VET Service GmbH, Austria) present in the home cages. Mice were acclimated in a reverse 12-h light and dark cycle (lights on at 6 PM and off at 6 AM) for a minimum of 7 days before the start of the study and kept in rooms with controlled temperature (20-22°C) and relative humidity (40-60%). Animals were housed in the same room in which the study was performed and had freely access to a rodent maintenance diet (Mouse and Rat Maintenance 3436 Kliba Nafag AG, Switzerland) and tap water. The facility is accredited by the Association for Assessment and Accreditation of Laboratory Care International (AAALACi).

Study design

Animals were randomly assigned with a prospective, blinded, parallel design to one of the administration groups [n= 3 females + 3 males/group; groups: intravenous (IV), intraperitoneal (IP), subcutaneous (SQ), orally per gavage (OS_{gavage}) as a single dose, oral in

drinking water (OS_{water}) and oral in Syrspend®SF PH4 Aroma-free (Fagron, Deutschland) (OS_{Syrsp}) for 25 hours].

All groups received 25 mg kg^{-1} of tramadol hydrochloride (Tramal® 100 injection solution, Grünenthal Group, Germany) either as a single dose or in drinking water or Syrspend®SF during 25 hours.

Due to the small volume required, a dilution of Tramal® 100 in NaCl 0.9% (B. Braun Medical AG, Switzerland) with a final concentration of 6.25 mg mL^{-1} was used for IV, IP, SQ and OS_{gavage} administration.

For T in drinking water or Syrspend®SF groups a solution of 0.20 mg mL^{-1} was prepared. Assuming an average daily water intake of 3.75 mL and 25 g BW, a solution of 0.20 mg mL^{-1} would ensure a dose of 25 mg kg^{-1} tramadol in 24 h (Harkness et al. 2013; Ingrao et al. 2013).

On the day of the experiment, the cage mates (n=3) were placed in metabolic cages and randomly assigned to one of the six treatments by drawing lots by the person administering the tramadol. A second operator, who was blinded to the administration routes, performed the blood collection at the determined time-points after drug administration; a volume of 50 µL of blood was drawn twice from each animal by tail-vein puncture with the conscious mice placed in a restrainer. The detail of the experimental set-up is depicted in Figure 1.

Insert figure 1.

All samples were collected into lithium-heparin-wetted tubes (POCT 50µl LH Minnivette, Sarstedt AG & Co, Germany) and stored in dry ice; plasma was separated by centrifugation at 3000 g for 10 minutes and frozen at -20°C until analysis (Musshoff et al. 2006; Cooper & Negrusz 2013).

Additionally, the stability of tramadol when diluted in different types of drinking water and Syrspend®SF was evaluated similar to the method described by Ingrao et al (2013). Tramadol (0.6 mL) was added to 150 mL water (bottle 1: non acidified water, F. Hoffmann-La Roche, Basel; bottle 2: non acidified water Unispital, Zürich; bottle 3: acidified water, F. Hoffmann-La Roche, Basel; bottle 4: acidified water, Unispital, Zürich) or Syrspend®SF (bottle 5), to achieve a final concentration of T (0.20 mg mL^{-1}). Water was non-chlorinated drinking-quality from the local water companies IWB (Basel-Stadt) and EWZ (Zurich). Full physico-chemical analysis was performed monthly and bacteriological quarterly (see Appendix 1). The acidified water was first filtered and demineralized and then acidified by adding HCl (ProMinent Dosiertechnik AG, Switzerland) until reaching a pH of 2.6-3.0.

All bottles were stored at ambient light and temperature conditions for up to 1 week. Samples were taken at 0, 24, 48 and 72 h and 7 days after the preparation of the solution and stored at -20°C until analysis (Musshoff et al. 2006; Cooper & Negrusz 2013).

Liquid chromatography and tandem mass spectrometry (LC-MS/MS)

Tramadol and M1 plasma concentrations were determined using liquid chromatography (LC) (Shimadzu Prominence, USA) coupled with mass spectrometry API 5500 (MS/MS) (ABSciex, USA). Plasma samples were processed using a protein precipitation procedure. A volume of 30 µl of methanol were added to 0.5 µl of plasma in 384 wells plate, agitated and centrifuged at 5600 rpm during 10 minutes. Then3 µl of supernatant were injected into the LC-MS/MS.

Mobile phase consisted of A: 10 mM Amonium acetate in water + 0.05% acetic acid (v:v), B: Methanol + 0.05% acetic acid (v:v). Flow rate was 0.8 mL min^{-1}. The run gradient started at 95%A / 5%B for 0.3 min then over 0.6 min, with a linear gradient to 20%A / 80%B, then over 1.2 min with a linear gradient to 5% / 95% B, returning to 95%A/ 5%B over 1.8 min with a linear gradient. Total run time was 3.9 min. Separation was achieved using a 2.1 x 30.0 mm column (C18 Ascentis express; Supelco, Sigma-Aldrich Corporation, Switzerland) maintained at 50°C.

Transition for tramadol was: parent ion (m/z) 263.9, daughter ion (m/z) 58.1; for M1: parent ion (m/z) 249.9, daughter ion (m/z) 58.1.

The analytical range for tramadol was 5-10000 ng mL^{-1} and for M1 was 25-10000 ng mL^{-1}. Standards extracted from spiked blank plasma gave calibration curves over the dynamic range. QC samples were run in replicates of 2 at a concentration of 5, 50 and 500 ng mL^{-1} for tramadol. Accuracy was assessed at each standard and QC level, all data points were within ±15% or ±20% at the lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ).

Pharmacokinetic and data analysis

Non-compartmental pharmacokinetic analyses (NCA) were performed with computer software (Phoenix WinNonlin Version 6.4; Pharsight Corp., CA, USA). The following pharmacokinetic parameters were calculated from the composite plasma concentration data after IV administration: the area under the curve (AUC_{last}) from time 0 to the last time point above the analytical LLOQ, the AUC extrapolated to infinity (AUC_{inf}), the percentage of the AUC_{last} extrapolated to infinity (AUC_{extrap}), plasma clearance (Cl), terminal half-life (t½), terminal rate constant (λz), volume of distribution at steady state (Vd), and apparent volume of distribution of the area during the elimination phase (Vz). The concentration at time 0 (C_0) was calculated by log linear back extrapolation using the first two time points after IV administration. The λz was determined using at least three time points. Pharmacokinetic parameters after extravascular administration (IP, SC, OS_{gavage}) from the composite plasma concentration data included the AUC_{last}, AUC_{inf}, t½, λz, plasma clearance per fraction of the dose absorbed (Cl/F), and apparent volume of distribution of the area during the elimination phase per fraction of the dose absorbed (Vz/F). The fraction of the dose absorbed (F) after extravascular administration was determined by dividing the oral AUC_{inf} by the IV AUC_{inf}. The maximum plasma concentration (Cmax) and time to maximum plasma concentration (Tmax) were determined directly from the plasma concentration data. The ratios of the tramadol and M1 AUC_{last} after different routes of administration were calculated where data permitted.

2.3 Results

In vitro

The measured concentration of the injectable tramadol (Tramal® 100) dilution in water (both acidified and non-acidified) and Syrspend®SF ranged from 0.193 mg mL^{-1} to 0.230 mg mL^{-1} (time 0 in Syrspend®SF was considered an artefact and therefore excluded), and remained stable for 7 days under ambient conditions (Fig. 2).

Insert figure 2.

The measured concentration of the injectable tramadol dilution in NaCl 0.9% (targeted to achieve a final concentration of 6.25 mg mL^{-1}) used for the parenteral and OS_{gavage} administration ranged between 6.24-6.34 mg mL^{-1} confirming the accuracy of the extemporaneous solution and was stable for 24 h under ambient conditions.

In vivo

After IV administration mild to moderate incoordination, ataxia, and *straub* tail were observed in all animals, the duration of these side effects being no longer than 40 seconds. In all other treatment groups no clinically evident side effect was observed.

Individual and mean plasma concentrations of tramadol and M1 were plotted versus time for each administration route (Fig. 3-6). The pharmacokinetic parameters for tramadol and M1 calculated from the composite concentration data for both genders combined are presented in Tables 1, 2 and 3.

Following IV administration, tramadol and M1 were quantifiable through 4 h and 2 h post-dose, respectively. Tramadol had high systemic clearance of approximately 160 mL/min/kg, large volume of distribution (Vd > 7.2L kg^{-1}), and short plasma half-life (<1 h). Exposure to M1, in terms of Cmax and AUC_{last}, was 15% and 20%, respectively, of the exposure to the parent drug.

Following IP administration, tramadol and M1 were quantifiable through 4 h and 2 h post-dose, respectively. Bioavailability for tramadol was 67%.

Following SQ administration, tramadol and M1 were quantifiable through 7 h post-dose. Systemic exposure in terms of AUC was comparable between the two routes. Furthermore, systemic exposure to M1 following SQ administration was ≥2-fold higher than M1 exposure after IV dosing, likely as a result from the prolonged absorption of the parent drug.

Following OS_{gavage} administration, tramadol and M1 were quantifiable through 7 h post-dose. Peak plasma concentration was reached at 1 h post-dosing. Oral bioavailability was 26% reflecting the extent of absorption and pre-systemic clearance due mostly to metabolism. Indeed, peak plasma concentration of M1 was comparable to that of the parent drug, while the metabolite to parent ratio was 1.3/1.5 for AUC_{last}/AUC_{inf}, respectively.

Insert tables 1 and 2

Apparent terminal half-life for the extravascular routes (1.4 h IP and 1.7 h OS_{gavage} and SQ) was twice the value of the estimated half-life after IV dosing and is suggestive of a flip-flop kinetics, e.g. absorption, rather than elimination, was the rate limiting step in tramadol systemic disposition and elimination, thus the estimated half-lives represent the absorption half-lives. The concentration profiles after IP and SC administration (Figure 3b and c) are suggestive of biphasic absorption and/or enterohepatic recycling, but no definitive conclusion could be made due to the limited number of animals and sampling time points.

The minimal tramadol plasma concentration for analgesic effect in humans has been estimated to be 100 ng mL^{-1} (Lewis & Han 1997), however, the threshold concentration in mice is not known. We used the 100 ng mL^{-1} concentration as a target level to achieve and maintain when comparing the dosing routes. After IV, IP or OS_{gavage} administration, tramadol

concentrations above and around 100 ng mL^{-1} were maintained over 2 to 4 h post-dosing, while, after SQ administration, values remained above 100 ng mL^{-1} for almost 6 hours.

Insert figures 3a-d and 4a-d.

After OS_{Syrsp} and OS_{water} administration, Cmax values were similar (328 and 315 ng mL^{-1}, respectively). With oral self-administration of tramadol diluted in Syrspend®SF, almost 6 hours were necessary to reach mean tramadol plasma levels above 100 ng mL^{-1}. Following this, mean tramadol concentrations remained stable (>200 ng mL^{-1}) until replacement of the drinking bottle with plain solutions. Conversely, mean tramadol concentrations above 150 ng mL^{-1} were reached after only 3 h when tramadol was diluted in water and remained above 100 ng mL^{-1} during consumption time (Fig. 5a-b).

High M1 concentrations (>100 ng mL^{-1}) where found in the OS_{water} group during consumption time, but 1 h after stopping the treatment, plasma concentration was half the concentration at 25 h. In the OS_{Syrsp} M1 could be detected only at one time-point, at 7 h (67.5 ng mL^{-1}) (Fig. 6).

Insert figures 5a-b and 6a-b.

Insert table 3.

Unfortunately, due to an unexpected leak in several bottles used, it was not possible to quantify water/Syrspend®SF intake as planned.

2.4 Discussion

The disposition and pharmacokinetics of a potentially clinically relevant dose of tramadol and its metabolite M1 after different routes of administration have been characterized for the first time in female and male B6 mice.

The plasma levels of both tramadol and M1 in B6 mice were high and in the analgesic range described for humans (Lehmann et al. 1990; Lewis & Han 1997) for up to 2 hours after IV with the greatest duration (6 hours) being after SQ administration. Therefore the expected analgesic effect of a single dose tramadol in this strain is likely to be of short duration depending on the administration route. Oral bioavailability after a single gavage dose was lower than that reported in other species (Lintz et al. 1986; Pypendop & Ilkiw 2008). However, when tramadol was administered in water or Syrspend®SF, the plasma levels achieved were high and remained stable during consumption time (25 h), which implies that animals kept voluntarily drinking the solution during that time, including their inactive phase during the day.

In compliance with the 3R principles (Russell et al. 1959; Carbone 2011), a sparse sampling design was used in this study with the minimal number of animals (N = 2 time points/gender). Following the recommendations of National Centre for the Replacement, Refinement and Reduction of Animals in Research (https://www.nc3rs.org.uk/mouse-decision-tree-blood-sampling), a maximum volume of blood of 0.01mL/mouse (<1% total blood volume) was collected in 24 hours. The data did not allowed to perform pharmacokinetic analysis beyond NCA, however, the results of the analysis are sufficient for assessing the exposure of the mice after different administration routes and prospective dose range finding and efficacy studies.

The dose tested in the present study was selected based on information obtained from a wide literature review and the extrapolation of doses from other species. For use in mice the Board for Anesthesia and Analgesia of the GV-SOLAS recommends the oral self-administration route with tramadol diluted in water at a concentration of 1mg mL^{-1} (Julia Henke 2015) resulting in a dose of 125 mg kg^{-1} of T in 24 h. However, no pharmacokinetic/ pharmacodynamic (PKPD) data are available to confirm that such a high dose is necessary and the higher the dose, the more likely to induce side-effects and bias research outcomes, which is highly undesirable and the #1 argument to avoid analgesic use in experimental mice (Gaspani et al. 2002).

Overall, the pharmacokinetic parameters of tramadol reported in the current study are in accordance with those obtained by Matthiesen et al. (1998) after 30 mg kg^{-1} tramadol orally given in mice: with high plasma concentrations (Cmax above 300 ng mL^{-1}), short half-life (<2 h) and similar AUC values. No comparisons can be made for the other administration routes.

Tramadol-induced analgesia results from both a monoaminergic and an opioidergic effect; the monoaminergic effect is activated mostly by tramadol, while M1 is the main responsible for the opioidergic effect, being up to 6 times more potent as analgesic as tramadol itself. Therefore, the metabolism of tramadol is key to its analgesic action. Tramadol is metabolized in the liver by the cytochrome p450 enzyme system and is responsible for the variability among species in the pharmacokinetics and metabolism (Raffa et al. 1993; Wu et al. 2001; Martignoni et al. 2006) and consequent analgesic efficacy of tramadol (Desmeules et al. 1996; Rukhshanda Saleem 2014). In the current study M1 concentrations paralleled tramadol concentrations after all administration routes, suggesting a rapid metabolism of tramadol to M1 in B6 mice. The faster metabolism in rodents compared to other species (Matthiesen et al. 1998; White & Seymour 2005) could explain the low bioavailability (F) found in mice in the current study (26%) after a single oral dose compared to humans (70%) (Lintz et al. 1986), horses 64% (immediate release capsules) (Giorgi et al. 2007) or cats (93%) (Pypendop & Ilkiw 2008). Nonetheless, a species-specific lower absorption of tramadol in the small intestine, contributing to a lower bioavailability cannot be excluded. Furthermore, results show a rather short half-life for both tramadol and M1 for mice in any of the routes tested compared to humans (Lewis & Han 1997). Due to the short elimination half-life observed, a frequent dosing will be required to maintain targeted plasma concentrations.

In humans, the minimal effective analgesic plasma concentration (MEC) of tramadol reported is 100 ng mL^{-1}, and for M1 a range of 39.6±29.5 ng mL^{-1} has been established (Lehmann et al. 1990; Lewis & Han 1997). In the present study, mean tramadol Cmax were above 300 ng mL^{-1} with all routes of administration and plasma concentrations of both tramadol and M1 were above the aforementioned minimal effective analgesic concentration range for humans. Hence, if the MECs reported in humans apply to mice also, it might be expected to achieve some level of analgesic effect with the dosage selected (25 mg kg^{-1}), the duration of which would vary according to the route, but this need to be assessed in an appropriate PKPD experimental setting.

In this study, although the highest plasma concentrations of both tramadol and M1 in mice were observed in the IV and IP groups, their concentrations decreased very fast and M1 concentrations were below the LLOQ after 2 h of the treatment, hence no analgesic effect after this time point is to be expected.

The most clinically relevant pharmacokinetic profile resulted after SQ administration: the targeted "potentially analgesic" plasma concentrations (T=100 ng mL^{-1} and M1=40 ng mL^{-1}) were extensively exceeded within 0.5 h and then maintained for as long as 6 hours. Therefore tramadol administered SQ could provide an early onset of and continuous antinociception for up to 6 h in B6 mice.

The half-life of tramadol and M1 in mice after a single dose is short, independently of the administration route, meaning that repetitive injections become necessary to achieve constant effective plasma levels over time. In laboratory rodents handling is associated with stress (Sharp et al. 2002; Jirkof et al. 2015) and should be avoided, not only for animal welfare but also as stress can bias the outcome data. Thus, self-administration methods or sustained release formulations are very attractive because they should ensure stable blood drug levels, while avoiding repetitive handling of the animals.

In addition to drinking water, non-flavored syrup (Syrspend®SF) was also selected as vehicle to potentially improve self-administration of the dissolved tramadol. Before use, the stability of tramadol in both vehicles for up to a week at ambient conditions was confirmed, warranting its practical use. Ingrao et al (2013) performed a stability analysis of meloxicam and carprofen in water in ambient dark, ambient light and 4°C dark conditions. However we intended to reproduce the same conditions that we find in our animal facility, thus testing our solutions in ambient light conditions was optimal for our purpose.

Mice were kept in a reverse 12 h light and dark cycle to match their peak circadian activity and hence fluid intake, providing more consistent data for the self-administration methods (OS_{water} and OS_{Syrsp}), Ingrao et al (2013).

Time to reach Cmax with auto-consumption was 3h and 7h for the mice drinking medicated water or the syrup suspension, respectively). Mice are a neophobic species (Kronenberger & Médioni 1985; Molina-Cimadevila et al. 2014) and the lack of habituation to Syrspend®SF before the beginning of the study might have contributed to this outcome, nevertheless a lower intestinal absorption rate of tramadol in Syrspend®SF could be of importance as well.

Plasma levels were high and constant over consumption time in both self-administration groups proving that tramadol in water and Syrspend®SF and at the dose chosen (25 mg kg^{-1} in 24 h), is palatable and well accepted by the animals. Unfortunately due to constant leaks from the bottle it was not possible to measure water/Syrspend®SF consumption in this setting.

An interesting finding was the detection of M1 only at the 7 h time-point in the OS_{Syrsp} group. After ruling out any analytical issues and a possible interference of any of the components of Syrspend®SF in the metabolism of tramadol, this observation remains currently unexplained. It is possible that Syrspend®SF – a food starch based vehicle- might slow the rate of intestinal absorption of tramadol. Thus, as tramadol is absorbed, it is transformed to M1 and eliminated without reaching measurable concentrations in plasma. This could account also for the delay in Tmax observed in this group, as no tramadol accumulation in blood occurs until hepatic saturation.

In the present study some mild and very transient side effects (incoordination and *straub*-tail) (Zarrindast et al. 2001), were observed after IV administration only and are in accordance with those found in the literature with seizures and sedation in mice and rats (Von G. Osterloch 1978; Raffa & Stone 2008; Cannon et al. 2010) and dizziness, headache, nausea and dry mouth in man (Lewis & Han 1997). Slow IV injection has been recommended to

avoid side effects following this route in humans and other species (Lewis & Han 1997; McMillan et al. 2008; Shilo et al. 2008); however in mice, this would be difficult to implement due to the small volume needed and the stress implied. In rats skin lesions following SQ administration (25 mg kg^{-1}) have been reported (Cannon et al. 2010), however this was not observed in any of the B6 mice.

In this study, females appear to have consistently lower concentrations of tramadol than males, except for the OS_{water} group, but a relevance of possible gender differences needs to be established in future studies with larger sample size. In addition, B6 mice were chosen because this is the most widely used strain in research, however further studies for the evaluation of possible strain differences would be of interest.

This study provides basic pharmacokinetic parameters for tramadol in B6 mice. 1) At the dose given, high plasma concentrations of both tramadol and its active metabolite M1 were obtained followed by a short half-life of 2 to 6 hours depending on the administration route. 2) Tramadol is stable in aqueous solutions for up to a week at ambient conditions and 3) self-administration of medicated water containing tramadol at a concentration of 0.20 mg mL^{-1} was successful in achieving constant plasma levels over consumption time.

Prospectively, this will allow selecting clinically relevant dose regimens for further pharmacodynamic testing in order to quantify objectively antinociceptive activity of tramadol in this mouse strain.

2.5 Tables

Table 1. Pharmacokinetic parameters for tramadol in B6 mice. Single 25 mg kg^{-1} T dose, administered IV, SQ, IP and oral (gavage). SE = Standard error; NA = Not applicable; NR = Not reportable (r^2<0.8). Results are presented with 3 significant figures.

Tramadol		**Dosing Route**			
PK Parameter (±SE)	**Units**	**IV**	**Oral**	**IP**	**SQ**
C_0	(ng/mL)	3450	NA	NA	NA
C_{max}	(ng/mL)	3710 (±1010)	347 (±170)	3010 (±410)	1870 (±575)
T_{max}	(h)	0.25	1	0.08	0.25
T_{last}	(h)	4	7	4	7
AUC_{last}	(h*ng/mL)	2580 (±265)	652 (±43.4)	1520 (±213)	2760 (±1060)
AUC_{inf}	(h*ng/mL)	2610	677	1750	2940
AUC_{extrap}	(%)	1.39	3.59	13.3	6.40
Cl or Cl/F	(mL/h/kg)	159	616	238	141
Vd	(mL/kg)	7,200	NA	NA	NA
Vz or Vz/F	(mL/kg)	10,100	89,400	27,700	20,400
t½	(h)	0.734	1.68	NR	1.67
F		NA	0.259	0.670	1.12

Table 2. Pharmacokinetic parameters for the active metabolite, O-desmethyltramadol (M1) in B6 mice (from composite data for genders combined). Single 25 mg kg^{-1} T dose, administered IV, SQ, IP and oral (gavage). SE = Standard error; NC = Not calculable. Results are presented with 3 significant figures.

M1		**Dosing Route**			
PK Parameter (± SE)	**Units**	**IV**	**Oral**	**IP**	**SQ**
C_{max}	(ng/mL)	549 (± 272)	313 (± 11.8)	487 (± 4.62)	735 (± 19.6)
T_{max}	(h)	1	0.5	0.25	0.5
$T_{last}t$	(h)	2	7	2	7
AUC_{last}	(h*ng/mL)	509 (± 233)	850 (± 208)	434 (± 33)	1630 (± 86.2)
AUC_{inf}	(h*ng/mL)	NC	1010	NC	2040
AUC_{extrap}	(%)	NC	15.9	NC	19.9
t½	(h)	NC	2.37	1.17	2.76
M1/T Ratio (C_{max})		0.148	0.902	0.162	0.393
M1/T Ratio (AUC_{last})		0.197	1.30	0.286	0.591

Table 3. Pharmacokinetic parameters for T and M1 in mice after self-administration in drinking water (from composite data for genders combined). Target dose of 25 mg kg^{-1} /24h based on water consumption of 3mL/animal/24h. M1 was quantifiable at a single time point for both genders and, therefore, no PK parameters were calculated reported. SE = Standard error.

Analyte	**Formulation**	**C_{max} (± SE)** (ng/mL)	**$AUC_{(0\text{-}25h)}$** (h*ng/mL)	**AUC_{last} (± SE)** (h*ng/mL)
Tramadol	T in Syrspend	315 (± 69.5)	5450	6170 (± 2240)
	T in water	328 (± 146)	4330	4630 (± 1940)
M1	T in water	658 (± 20)	6280	6280 (± 239)

2.6 Figures

Figure 1. Sampling set-up: 3 mice/ gender were allocated to each administration group and two 50 µL samples/mouse were collected. These data points were used to create a composite PK profile.

a)

Mouse #		Sampling times post SQ/Oral (gavage) dosing					
♀	♂	15 min	30 min	1 h	2 h	4 h	7 h
1	4	x			x		
2	5		x			x	
3	6			x			x

b)

Mouse #		Sampling times post IP/IV dosing					
♀	♂	5 min	15 min	30 min	1 h	2 h	4 h
1	4	x			x		
2	5		x			x	
3	6			x			x

c)

Mouse #		Sampling times from start of drinking water /Syrspend® self-dosing					
♀	♂	1 h	3 h	7 h	25 h*	26 h	31 h
1	4	x			x		
2	5		x			x	
3	6			x			x

* replaced with plain solution

Figure 2. Stability of tramadol HCl injectable solution (T; Tramal® 100, 50 mg mL^{-1}) in Sysrpend®SF, Basel acidified, Basel non-treated, Zurich acidified and Zurich non-treated water and held at ambient conditions for 7 days.

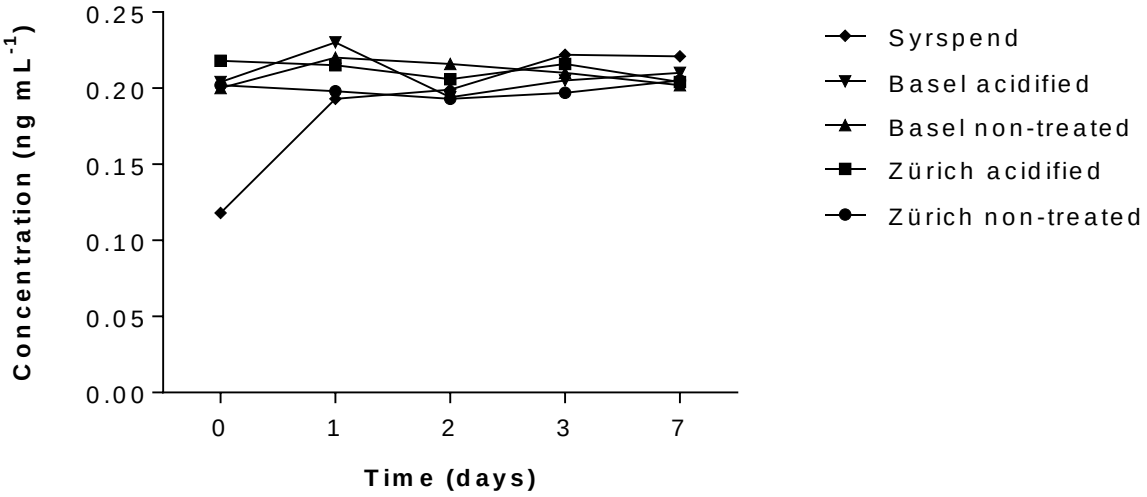


Figure 3. Semi-log composite plots of female, male and mean plasma T concentrations over time after a) IV, b) IP, c) SQ and d) OS_{gavage} administration of 25 mg kg^{-1} T. The horizontal line represents the minimal effective analgesic plasma level of T (100 ng mL^{-1}) in humans. Females, males, mean.

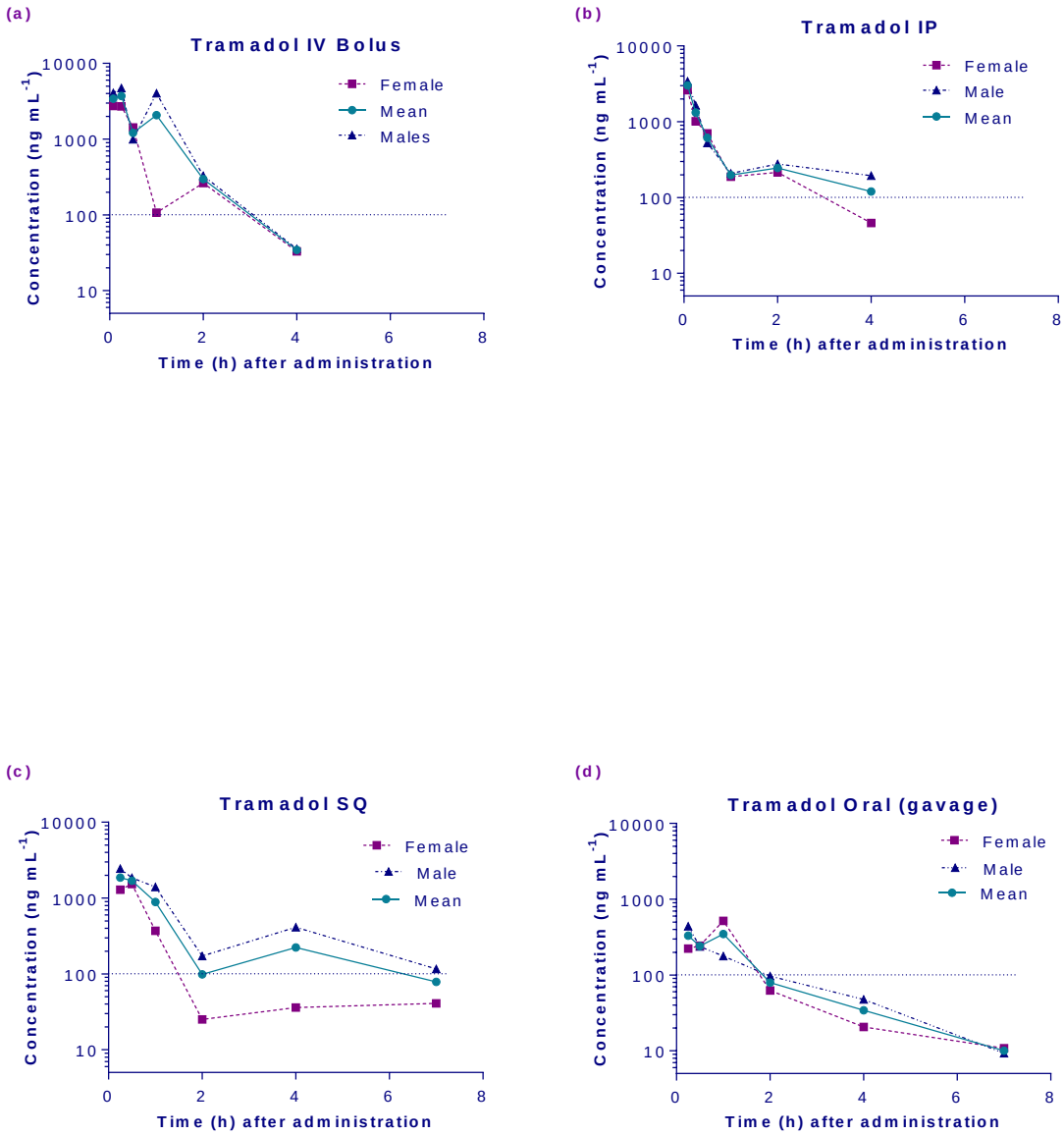


Figure 4. Semi-log composite plots of female, male and mean plasma M1 concentrations over time after a) IV bolus, b) IP, c) SQ and d) OS_{gavage} administration of T. The horizontal line represents the minimal effective analgesic plasma level of M1 (40 ng mL^{-1}) in humans. After 25 h (arrow) the drinking water/Syrspend®SF were replaced with plain solutions. Females, males, mean.

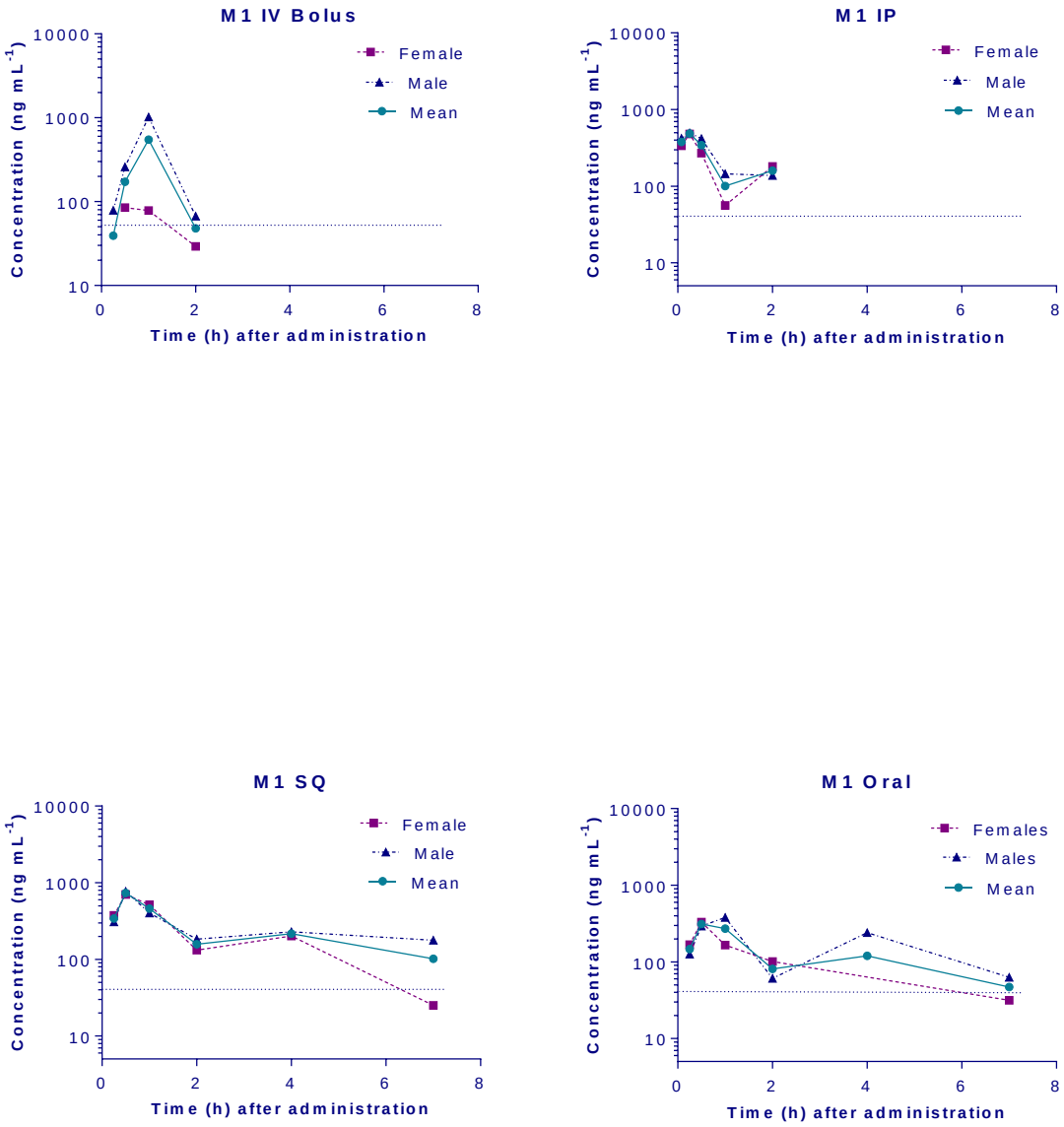


Figure 5. Semi-log composite plots of female, male and mean plasma T concentrations over time in a) OS_{water} and b) OS_{Syrsp} administration of 25 mg kg^{-1} T over 25 h. After 25 h (arrow) the drinking water/ Syrspend®SF were replaced with plain solutions. The horizontal line represents the minimal effective analgesic plasma level of T (100 ng mL^{-1}) in humans. Females, males, mean.

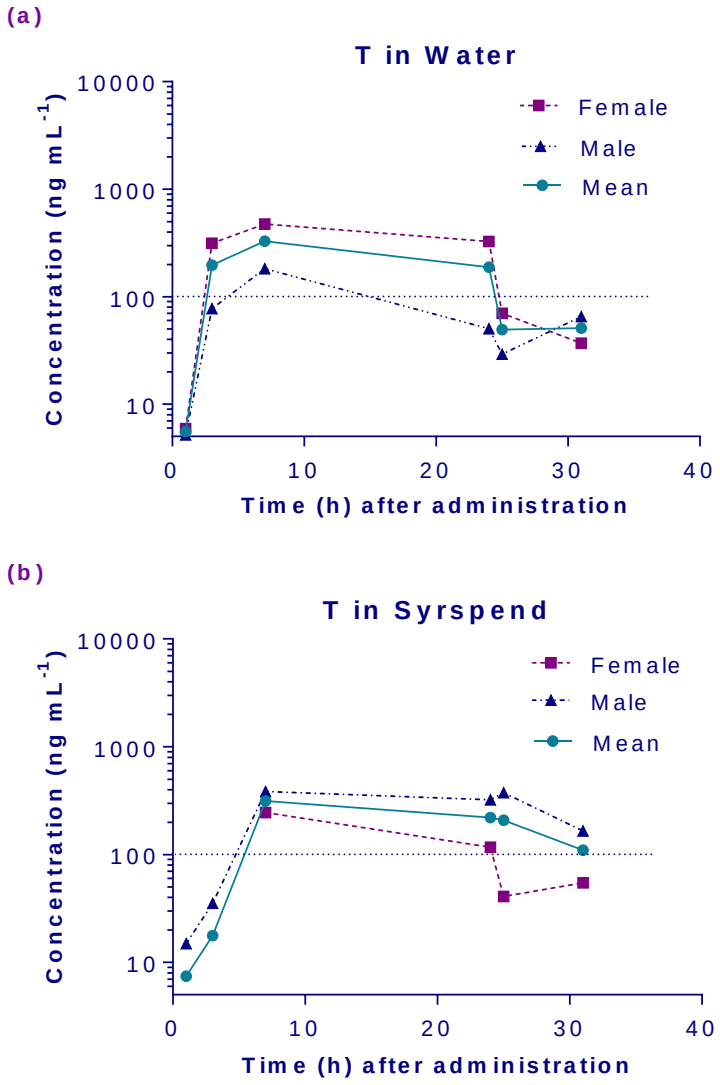
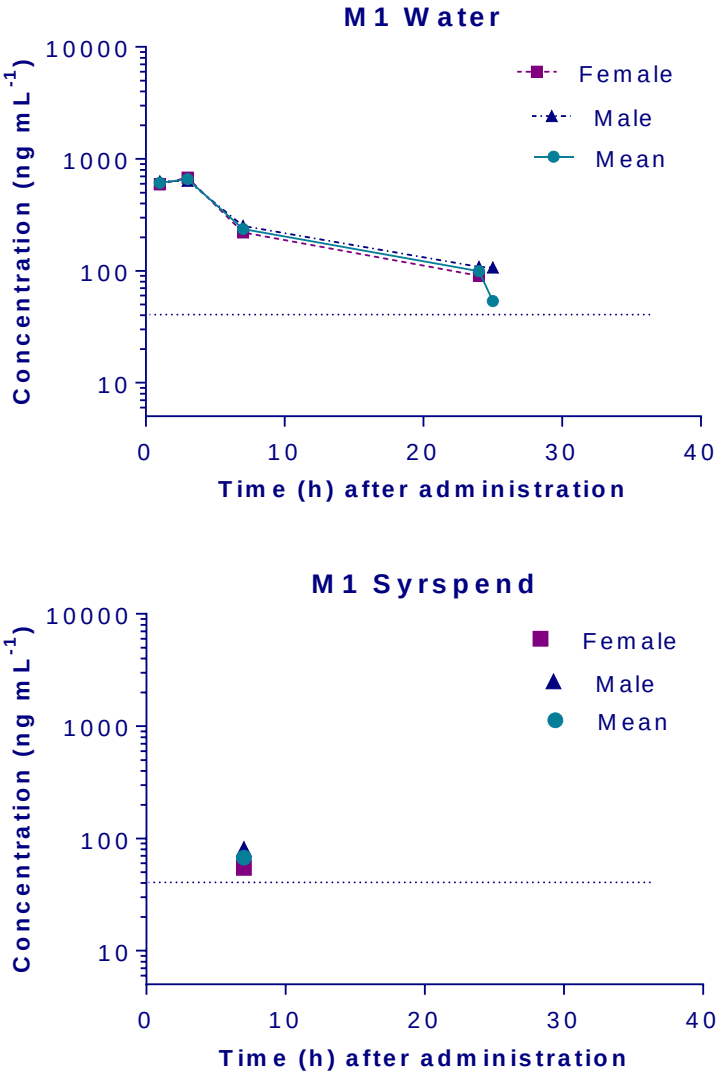


Figure 6. Semi-log composite plots of female, male and mean plasma M1 concentrations over time in a) OS_{water} and b)OS_{Syrsp} groups. After 25 h (arrow) the drinking water/Syrspend® SF were replaced with plain solutions. The horizontal line represents the minimal effective analgesic plasma level of M1 (40 ng mL^{-1}) in humans. Females, males, mean.



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